Vol. 201, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
May 30, 1994 Pages 16-23

SALMONELLA TYPHIMURIUM ACTIVATES HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 IN CHRONICALLY INFECTED PROMONOCYTIC CELLS BY INDUCING TUMOR NECROSIS FACTOR-α PRODUCTION

Augusto Andreana, Sastry Gollapudi*, Choong H. Kim, and Sudhir Gupta

Division of Basic and Clinical Immunology, University of California, Irvine, CA 92717

Received March 28, 1994

The effect of phagocytosis of *Salmonella typhimurium* on human immunodeficiency virus type 1 (HIV-1) production was investigated using a chronically infected promonocytic cell line (U1) that contains HIV-1 provirus but produces little or no HIV-1. The phagocytosis of virulent *S. typhimurium* by U1 cells resulted in an increased HIV-1 expression as evidenced by significant increase in HIV-1 p24 antigen in culture supernatants. In contrast, heat-killed *S. typhimurium* failed to induce HIV-1 expression. In addition, phagocytosis of virulent *S. typhimurium* and not of heat-killed *S. typhimurium* resulted in a significant induction of tumor necrosis factor-α (TNF-α) mRNA expression and secretion of TNF-α by U1 cells. Furthermore, anti-TNF-α monoclonal antibody inhibited *S. typhimurium*-induced HIV-1 p24 antigen production. These data suggest that *S. typhimurium* induces HIV-1 expression in U1 cells via production of TNF-α.

Reactivation of latent Human immunodeficiency virus (HIV) is thought to play a role in the pathogenesis of the acquired immunodeficiency syndrome (1). *In vitro* studies have demonstrated that co-infection of HIV infected cell lines with certain DNA viruses (2,3), *Mycobacterium tuberculosis* (4) and *mycoplasma* (5) resulted in reactivation of latent HIV. In addition, it has been shown that cytokines produced by activated lymphocytes and monocytes such as tumor necrosis factor α (TNFα) can induce HIV expression from a state of latent or chronic infection to productive viral expression (6). These studies have suggested that secondary infections with other microbes in HIV infected individuals could activate HIV *in vivo* and accelerate disease process.

An increased incidence of bacteremic *Salmonella* infection, which many at time precedes the diagnosis of the acquired immunodeficiency syndrome (AIDS), was recognized

* Correspondence should be addressed to Dr. Sastry Gollapudi, Med. Sci. I, Room C-240, University of California, Irvine, CA 92717. FAX: (714) 856-4362.

0006-291X/94 $5.00

early in the epidemic (7). *Salmonella* infection in HIV-1 infected subjects differs in several important aspects from that in non-AIDS individuals (7,8). First, *Salmonella* infection is approximately 20 fold more common in HIV-1 infection as compared to non-HIV-1 infected subjects (7); second, the incidence of *Salmonella* bacteremia in HIV-1 infected individuals is higher than non-AIDS individuals; and third, the bacteremia may be persistent or recurrent despite treatment with appropriate antimicrobial therapy. In this study we examined the effect of phagocytosis of virulent and avirulent *S.typhimurium* on HIV expression and on production of TNF α in chronically HIV infected promonocytic U1 cells that produce little or no virus. The results presented here demonstrate that virulent *Salmonella* stimulate HIV production in U1 cells largely via production of TNF-α.

MATERIALS AND METHODS

Cell line: The chronically HIV-1-infected cell line U1, a subclone of HIV-1- infected U937 promonocytic cell line (U937/HIV), was obtained from the AIDS Research and Reference Program of the National Institutes of Health, Rockville, MD. The U1 subclone differs from the parent U937/HIV cell line in that U1 cells express HIV-1 provirus but produce minimal or no HIV-1 virion (9). Therefore, U1 cell line provides an *in vitro* model to study the mechanisms of HIV-1 induction/modulation. U1 cells were maintained in RPMI-1640 medium supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS, Hyclone, Logan,UT) and antibiotics.

Salmonella typhimurium: *S. typhimurium* (strain ATCC 14028) was obtained from the American Tissue Culture Collection, Rockville, MD and processed with standard microbiological technique. An overnight culture of *S. typhimurium* on brain heart infusion (DIFCO, Detroit, MI) at 37^{0}C produced an average of 0.6-1.2 x 10^9 bacteria/ml. The bacteria were washed three times with phosphate buffer saline (PBS), resuspended in RPMI-1640 medium supplemented with FBS, and used at 1:10 dilution. Heat-killed *S. typhimurium* was prepared by heating virulent bacteria at 60^{0}C for 30 minutes. Aliquots of these bacteria were stored at -90^{0}C until used.

ELISA kits: HIV-1 p24 antigen in the supernatants was measured by antigen-capturing ELISA assay using commercial kit (Retro-Tek ELISA kit, Cellular Products, Inc., Buffalo, New York). TNF-α was also measured by ELISA assay (Endogen, Boston, MA).

Anti-tumor necrosis-α antibody: Anti-TNF-α monoclonal antibody was a gift from Dr. Bharat Aggarwal, M.D. Anderson Cancer Center, Houston, TX.

Tumor necrosis-α factor primers: To detect TNFα mRNA by PCR, following primers were synthesized by ChemGene, Waltham, MA.: TNF-α, 5'ATGAGCACTGAAAGCATGATC-3' (sense) and 5'-TCACAGGGCAATGATCCCAAAG-3' (antisense) and control β-actin, 5'-GTGGGGCGCCCCAGGCACCA-3' (sense) and 5'-CTCCTTAATGTCACGCACGAT-3' (antisense).

Phagocytosis of S. typhimurium by U1 cells: An inoculum of 1 x 10^8 virulent or heat-killed opsonized *S. typhimurium* was added to 1 x 10^7 U1 cells in 3 ml of RPMI-1640 medium (supplemented with 10% human AB serum without antibiotics) and incubated for 20 minutes at 37^{0}C. The growth of extracellular bacteria was inhibited by an addition of gentamicin to the culture medium (10) A sample of cells was centrifuged on a slide by cytospin (Shandon, Philadelphia, PA), stained with Giemsa stain and phagocytosis of bacteria was confirmed by light microscopy. Cells were washed three times with PBS, resuspended in medium, transferred to tissue culture flask and incubated at 37^{0}C for various time intervals.

Cultures were centrifuged and supernatant was collected and stored at -90°C until assayed for HIV-1 p24 antigen and TNF-α levels. Cells were used for the extraction of RNA for TNF-α mRNA assay. The virulent *S. typhimurium* survived in U1 cells during all three days of culture as assessed by enumeration of colony forming units of bacteria grown on agar plates after lysing the cells with 0.5% deoxycholate.

Effect of anti-TNF-α antibody on HIV-1 production: In order to determine whether *S. typhimurium*-induced HIV-1 expression in U1 cells is via production of TNF-α, U1 cells that have phagocytized virulent *S. typhimurium* were incubated at 37°C in the presence or absence of neutralizing concentration of anti-TNF-α monoclonal antibody. Culture supernatants were collected at different time intervals and assayed for HIV-1 p24 antigen and TNF-α (the latter to determine the level of TNF-α neutralized by the antibody).

Tumor necrosis factor-α mRNA: U1 cells phagocytosed with virulent or heat-killed *S. typhimurium* were cultured for 2 hours and TNF-α mRNA was detected by reverse transcriptase based polymerase chain reaction (RT-PCR), using a kit from Perkin-Elmer (Norwalk, CT). Cellular RNA was extracted by guanidinium thiocyanate-phenol chloroform method (11). Two hundred nanograms of total RNA was used as a template for cDNA synthesis and amplification by PCR. Amplification was done at 94°C for 1 minute, at 60°C for 1 minute, and 72°C for 2 min for 35 cycles.

Statistical analysis was performed by paired student t test.

RESULTS

Phagocytosis of virulent S. typhimurium induces HIV-1 production: Control U1 cells and U1 cells that have phagocytized virulent or heat-killed *S. typhimurium* were incubated at 37°C for various time intervals, supernatants collected and assayed for HIV-1 p24 antigen using ELISA kit. Results are shown in Table 1. Control U1 cells produced negligible amounts of HIV-1 p24 antigen (peak at day 3; 102 ± 15 pg/ml). Virulent *S. typhimurium*, in a time-dependent manner, induced significant levels of HIV-1 p24 antigen (peak at day 3; 1949 ± 134 pg/ml). In contrast, heat-killed *S. typhimurium* induced very low levels of HIV-1 p24 antigen (peak at day 3; 348 ± 134 pg/ml). The levels of HIV-1 p24 antigen induced by heat-killed *S. typhimurium* were significantly lower ($P<0.01$) as compared to those induced by virulent *S. typhimurium*. The phagocytosis of virulent and heat killed

Table 1
Effect of *S. typhimurium* on HIV-1 p24 antigen production by U1 cells

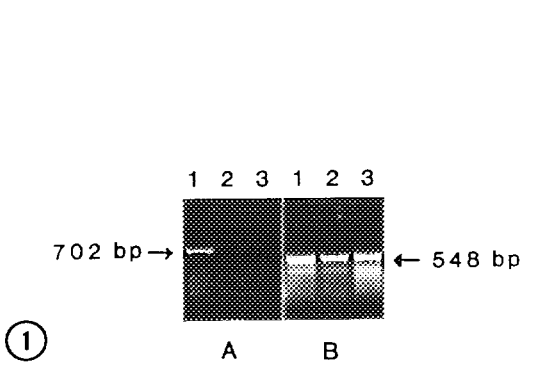
Exp. Condition	HIV-1 p24 antigen (pg/ml)		
	Day 1	Day 2	Day 3
U1 Cells	47 ± 3	67 ± 9	102 ± 15
U1 + HKST	ND	230 ± 96	348 ± 134
U1 + VST	617 ± 103	1075 ± 170	1949 ± 360

U1 cells were incubated with or without virulent *S. typhimurium* (VST) or heat-killed *S. typhimurium* (HKST) for 2 hours, washed and then incubated further for 3 days. Culture supernatants were collected on day 1, day 2 and day 3 for the measurements of HIV-1 p24 antigen by ELISA. Data are expressed as mean ± SD of three separate experiments. ND= not done.

S.typhimurium by U1 cells was comparable (data not shown). Therefor the lack of induction of HIV-1 p24 antigen by heat-killed *S. typhimurium* would exclude any significant role of the process of phagocytosis in the induction of HIV-1 expression by virulent *S. typhimurium*.

Phagocytosis of virulent S. typhimurium induces TNF-α production: U1 cells with or without virulent or heat-killed *S. typhimurium* were incubated at 37°C for various time intervals. Cultures were centrifuged and supernatants collected for the measurements of secreted TNF-α by ELISA assay. Cellular RNA was extracted and used for the analysis of TNF-α mRNA by RT PCR. The results of PCR analysis of TNF-α mRNA are shown in Figure 1. Virulent *S. typhimurium* increased TNF-α mRNA to a much greater extent (Lane 1) than induced by heat-killed bacteria (Lane 2). Data of secreted TNF-α are shown in Figure 2. Control U1 cells and U1 cells with heat-killed *S. typhimurium* produced very low levels of TNF-α (<30pg/ml). By contrast, U1 cells with virulent *S. typhimurium* produced significantly higher ($P<0.01$) levels of TNF-α (326 pg/ml). These data demonstrate that virulent *S. typhimurium* induces TNF-α expression by both transcriptional and translational mechanisms.

Anti-TNF-α monoclonal antibody inhibits S. typhimurium-induced HIV-1 expression: Because virulent *S. typhimurium* induced both HIV-1 and TNF-α expression, we investigated whether there was a direct correlation between the induction of TNF-α and an activation of HIV-1 by *S. typhimurium*. U1 cells with virulent *S. typhimurium* were cultured in the presence or absence of anti-TNF-α monoclonal antibodies for different time intervals and culture supernatants were analyzed for HIV-1 p24 antigen and TNF-α levels. Figure 3



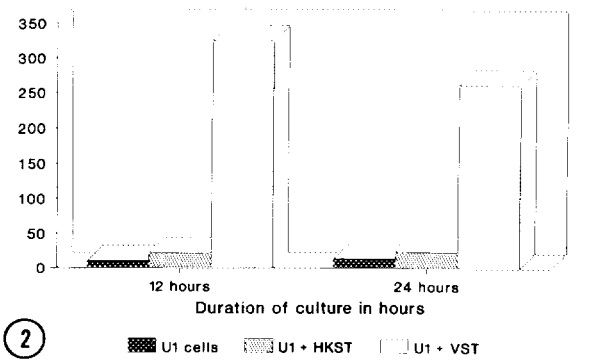


Figure 1. Effect of phagocytosis of live or heat killed *S. typhimurium* on the expression of TNF-α. (A)TNF α mRNA in U1 cells (lane 3), U1 cells with heat-killed *S. typhimurium* (lane 2), and U1 cells with virulent *S. typhimurium* (Lane 1). Control β-actin is shown in B.

Figure 2. Effect of phagocytosis of live or heat killed *S. typhimurium* on Tumor necrosis factor (TNF-α) production. Data are expressed as mean pg/ml of three separate experiments.

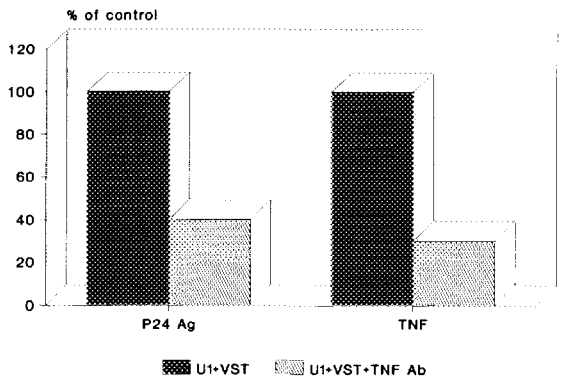


Figure 3. Effect of anti-TNF-α monoclonal antibody on *S. typhimurium*-induced HIV-1 p24 antigen production and neutralization of released TNFα. Culture supernatants of U1 cells incubated without anti TNF α antibody yielded 1949 pg/ml of HIV-1 p24 and 228 pg/ml of TNF α. Culture supernatants of U1 cells incubated with anti-TNF α yielded 919 pg/ml of HIV-1 p24 and 69 pg/ml of TNF-α.

shows that anti-TNF-α antibody at a concentration that neutralized TNF-α by 70% (from 228 pg/ml to 69 pg/ml) also inhibited HIV-1 p24 antigen production by 53% (from 1949 pg/ml to 919 pg/ml). These data suggest that the major mechanism of *S. typhimurium*-induced HIV-1 expression is via TNF-α production.

DISCUSSION

In the present study we have observed that TNF-α is a crucial mediator of *S. typhimurium* induced HIV-1 replication in chronically HIV infected promonocytic U1 cells. The phagocytosis of live virulent *S.typhimurium* and not of the heat killed *S. typhimurium* by U1 cells resulted in increased HIV P-24 antigen and TNF-α mRNA expression and TNF-α production.

One of the features of HIV-1 infection is the long incubation period (approximately 10-12 years) during which the virus is harbored in a latent state in CD4+ target cells, especially in cells of monocyte/macrophage lineage (12). The acquisition of secondary infections in HIV-1 positive individuals leads to rapid progression of the disease (13). Therefore, a number of agents have been considered as "co-factors" that could accelerate the progression of disease by upregulating HIV-1 replication. This has been particularly true with human cytomegalovirus (HCMV), human herpes simplex virus (HSV), and *Mycobacterial* species infections (2-4). Pettoello-Mantovani et al (14) reported enhancement of HIV-1 by capsular polysaccharide of *Cryptococcus neoformans* in T cell line and lymphocytes from HIV-1 infected patients. In the present study we showed that phagocytosis of virulent strain

of *S. typhimurium* induces HIV-1 replication in a chronically infected U1 cells that otherwise produce little or no HIV-1.

Conflicting data have been reported regarding the effect of phagocytosis of bacteria on HIV production. The phagocytosis of *M. tuberculosis, M.avium intracellulare,* killed *Staphylococcus aureus, Escherichia coli,* erythrocytes, zymosan or latex particles resulted in either no effect or decrease in HIV production by acutely infected human peripheral blood derived macrophages (15-18). In contrast Shattock et al (4) showed that phagocytosis of *M. tuberculosis* by latently HIV infected mature monocytic leukemia cells stimulate HIV production. Our results are in agreement with that of Shattock et al (4). The discrepancy on the effect of phagocytosis on HIV production may be related to differences in target HIV infected cells used. Alternatively, it is possible that the effect of phagocytosis of microbes on HIV activation may depend on the stage of HIV infection (latent vs acute) . Furthermore, the nature and type of the microorganism may also contribute to some of the differences. For example, Friedland et al (19) reported that the phagocytosis of *M. tuberculosis* and not of *Toxoplasma gondii* by THP-1 cells, a human monocytic cell line, resulted in increased TNF-α expression.

During the past 5-6 years the role of cytokines in the regulation of HIV-1 expression has been investigated. The following evidence are in support of the role of cytokines in HIV-1 regulation: [a] increased plasma or cerebrospinal fluid levels of TNF-α, IL-1, and IL-6 in HIV-1 positive patients as compared to uninfected individuals (20,21); [b] increased *in vitro* production of TNF-α and IL-6 by stimulated PBMNC from HIV-1 seropositive subjects (22,22); [c] production of TNF-α and stimulation of HIV-1 expression in chronically infected cell line by phorbol myristate acetate (9,23) or LPS (24,25); and [d] upregulation of HIV-1 expression in chronically infected cells by TNF-α and IL-6 (1,26). Peterson et al (27) also reported that TNF-α was the key mediator of HCMV-induced HIV-1 replication in U1 cells and acutely HIV-1-infected PBMNC. In the present study we also show that phagocytosis of virulent typhimurium resulted in increased TNF-α mRNA and production of TNF-α. Furthermore, we observed a correlation between the degree of co neutralization of TNF-α (70%) by anti-TNF antibody and the extent of inhibition of P24 antigen (53%). Although we did not detect mRNA for IL-1β or IL-6 in U1 cells phagocytosed with *S. typhimurium* (data not shown), the role of other cytokines in our experimental conditions can not be excluded. Tumor necrosis factor-α has been shown to induce HIV-1 gene expression in chronically infected cell lines by a transcription mechanism involving nuclear factor-*k*B (24,25). Studies are currently in progress to determine whether similar mechanisms are involved in *S. typhimurium*-induced HIV-1 replication.

The clinical significance of our in vitro results remains to be established. At present no data are available on HIV titers prior to and post infection with *Salmonella* in patients with AIDS. Epidemiological studies are needed to determine whether *salmonella* infection in HIV seropositive patients results in HIV disease progression.

ACKNOWLEDGMENTS

U1 cells were obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH from Dr. Thomas Folks (9). This work was supported by a grant from the University of California Universitywide AIDS Research Program (R92-I-100).

REFERENCES

1. Pantleo G., Grazioli G., & Fauci A.S. (1993). New. Eng. J. Med. 328: 327-335.
2. Casareale D., Fiala M., Chang C.M., Cone L.A., & Mocarski E.S. (1989). Int. J. Cancer 44: 124-130.
3. Tremblay M., Gornitsky M., & Weinberg M.A. (1989) J. Med. Virol. 29: 109-114.
4. Shattock R.J., Friedland J.S., & Griffin G.E. (1993). Rev. Virol. 144: 7-12.
5. Lo S.C., Tsai S., Benish J.R., Shih J.W., Wear D.J., & Wong D. (1991). Science. 251: 1074-1076.
6. Fauci A.S (1990). Lymphokine Res. 9: 527-531.
7. Sperber S.J. & Schleupner C.J. (1987). Rev. Infect. Dis. 9: 925-934.
8. Profeta S., Forrester C., Eng R.H.K., Liu R., Johnson E., Palinkas R., & Smith S.M. (1985). Arch. Int. Med. 145: 670-672.
9. Folks T.M., Justment J., Kinter A., Dinarello C.A., & Fauci A.S. (1987). Science 238: 800-802.
10. Tabrizi S.N. & Robbins-Browne R.M. (1993). J. Immunol. Methods. 158: 201-206.
11. Choczynski P. & Sacchi N. (1987). Analyt. Biochem. 162: 156-159.
12. Gendleman H.E., Orenstein J.M., Kalter D.C. & Roberts C. (1992). In AIDS and Other Manifestations of HIV Infection. G.P. Wormser Ed. Raven Press, N.Y.p57-75.
13. Haverkos H.W.(1987). J. Inf. Dis. 156: 250-257.
14. Pettoello-Mantovani M., Casadevall A., Kollmann T.R., Rubenstein A., & Goldstein H. (1992). Lancet 339: 21-23.
15. Meylan P.R.A., Munis J.R., Richman D.D. & Kornbluth R.S. (1992). J. Infect. Dis. 65:80-86.
16. Newman G.W., Kelley T.G., Gan H., Kandil O., Newman M.J., Pinkston P., Rose R.M., & Remold H.G. (1993). J. Immunol. 151:2261-2272.
17. Nottet H.S.L.M., de Graaf L., Machiel de Vos N., Bakker L.J., van Strijp J.A.G., Visser M.R., & Verhoef J. (1993). J. Infect. Dis. 167:810-817.
18. Piedmonte G., Montroni M., Silvestri G., Silvotti L., Donatini A., Rossi L., Borghetti A.F., & Magnani M. (1992). Arch. Virol. 130:463-469.
19. Friedland J.S., Shattock R.J., Johnson J.D., Remick D.G., Holliman R.E., & Griffin G.E. (1993). Clin. Exp. Immunol. 91: 282-286.
20. Breen E.C., Rezai A.R., Nakajima K., Beall G.N., Mitsuyasu R., Hirano T., Kishimoto T., and Martinez-Maza O. (1990). J. Immunol. 144: 480-484.
21. Lahdevirta J., Maury C.P.,Teppo A.M. & Repo H.(1988). Amer.J.Med. 85:189-192.
22. Merrill J.E., Koyanagi Y., & Chen I.S.Y. (1989). J. Virol. 63: 4404-4408.
23. Folks T.M., Justment J., Kinter A., Schnittman S., Orenstein J., Poli G. & Fauci A.S. (1988). J. Immunol. 140: 1117-1122.

24. Lantham P.S., Lewis A.M., Varesio L., Pavlakis G.N., Felber B.K., Ruscetti F.W. & Young H.A. (1990). Cell. Immunol. 129: 513-518.
25. Pomerantz R.J., Feinberg M.B., Trono D., & Baltimore D. (1990). J. Exp. Med. 172: 253-261.
26. Osborn L., Kunkel S., & Nabel G.L. (1989) Proc. Natl. Acad. Sci.(USA). 86:2336-2340.
27. Peterson P.K., Gekker G., Chao C.C., Hu S., Edelman C., Balfur Jr. H.H., & Verhoef J. (1992). J. Clin. Invest. 89: 574-580.